Age-associated decline in phosphorylated Connexin 43 protein expression in the left ventricular tissue of Wister rats.

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Abstract

Cardiac arrhythmia affects ~ 6% in those over 65 years of age (old), but with 0.2% occurrence in those of 45 years and below (young). Arrhythmia can result from dysregulation of the cardiac impulse generation and its conduction. Connexin proteins are responsible for cardiac impulse conduction, and phosphorylation of connexin 43 determines its functional ability. In this study, Phosphorylated connexin 43, density and expression were assessed in ventricular tissues from young (6 months old) and old (24 months old) Wister rats, using the techniques of western blot and immunohistochemistry. Results show that phosphorylated Cx43 in the left ventricle of 24 months old rats significantly declined (P=0.04 & 0.01) by method of western blot and immunohistochemistry respectively, but did not differ in the right ventricle. The left ventricle is known to be responsible for cardiac output. This data suggest an age-associated decline in the expression of phosphorylated connexin 43 in the left ventricle, which may play a significant role in the development of cardiac arrhythmia in the elderly.

Introduction

In the aged population (65 years and above), cardiac arrhythmia is a leading cause of hospital admissions and eventual death [1, 2, 3]. The mechanism responsible for cardiac arrhythmias, although multifactorial, can be broadly subdivided into disorders of impulse formation (automaticity) and those of impulse conduction (re-entry) [3, 4]. One of the major causes of re-entrant cardiac arrhythmia is altered coupling of cardiac myocytes because of the alteration in the expression and localization of connexin (Cx) proteins [5, 6], resulting in a change in anisotropy [7].

Connexin proteins are gap junction proteins that connect two adjacent myocytes, enabling the transmission of electronic impulse and low molecular weight substances (≤ 1kD) between the two myocytes. This transfer of electronic impulses enables the myocardium to contract and relax in unison (syncytium). There are different types of connexin proteins occurring principally in different vertebrate tissue types. Cx43 was the first connexin to be identified and is most abundantly expressed in different organs and cell types of animal and human tissue; it is also the most abundant protein of the gap junctions in the cardiac tissues. In the heart, Cx 43 is more abundant in the ventricles, Cx45 in the sinoatrial and atrioventricular nodes while Cx40 is more abundant in the atrial regions of the heart.

The association of cardiac connexins with cardiac arrhythmia has been documented. Investigators have shown a decline in Cx43 expression in the myocardium of subjects with increased tendency to develop arrhythmia [8, 9]. In arrhythmogenically remodelled hearts, Cx43 is often down-regulated, with increased lateralisation and less phosphorylation [10, 11 and 12]. Phosphorylation reactions occurring in proteins is commonly considered to be the most common post translational modification modulating their function and differential phosphorylation of connexin proteins determines their localization and ability to form functional gap junctions [13,14].

The phosphorylated forms of Cx43 constitute about 85% of total Cx43 density in the cardiac tissues. Protein kinase C (PKC) activation, extracellular signal-regulated kinase (ERK), and tyrosine v-Src kinase phosphorylation of Cx43 result in diminished total conductance and trans-junctional communication between adjacent cardiomyocytes [15, 16, 17]. Calmodulin kinase II (CAMKII) phosphorylates S244, S314, S296, S297 & S306 of Cx43 resulting in delayed onset of ischemic induced arrhythmias [18,19], while mitogen activated protein kinase (MAP kinase) activation at S255, S279 & S282 has been documented to disrupt the growth of gap junction plaque [20,21,22]. Lateralization of connexins 43 correlates with the dephosphorylation of serine S325, S328 and S330 with a resultant effect of conduction velocity slowing and increased potential for arrythmogenicity [23, 24]. Following the increased preponderance of diseases of cardiac conduction defects in the elderly population, it is unknown if the density and expression pattern of phosphorylated Cx43 protein in the elderly is different from the young. This work is therefore designed to investigate the density and expression pattern of phosphorylated Cx43 in the young compared to the elderly. The result of this work will contribute to the knowledge of factors responsible for increased occurrence of cardiac arrhythmia in the elderly.

Materials & Methods

Tissue acquisition

Wister rats aged 6 and 24 months were acquired from Charles River Inc, Kent, and maintained per United Kingdom Home Office regulations until experimentation. The Wister rats were sacrificed by schedule 1 (overdose of sodium pentobarbitone) in accordance with the Animals (scientific Procedures) act 1986 as amended in 2012.

Wister rats were weighed and dissected to obtain the hearts. The hearts were blotted of excess blood and rinsed in oxygenated bicarbonate–buffered tyrode solution at 37 ^{0}C, and then submerged in cold cardioplegic solution (Glucose 227.5 mM, Potassium chloride 30 mM, sodium hydrogen carbonate 25 mM and Mannitol 34.3 mM) for 10 minutes. From the cardioplegic solution, the hearts were transferred to a silicone based plastic dish containing 37 ^{0}C oxygenated bicarbonate-buffered tyrode solution and dissecting pins were used to hold the heart in place. An incision was made across the heart to dissect the atria away from the ventricles. Then another incision was made along the ventricular septum to dissect away the right and left ventricles, trimmed into small pieces and stored at -80 ^{0}C until used for analysis.

Western blot

Tissue lysates were made in homogenising buffer composed of 1mM iodoacetamide, 1mM benzothonium chloride, 5.7 phenylmethylsulfony fluoride and 1% SDS, 10mM EDTA, and 300 mM sucrose. Protein concentration was determines by Pierce BCA protein assay (ThermoFisher 23225) and 60 µg of proteins were separated on 10% SDSPAGE. Proteins were transferred onto 0.45µm pore size nitrocellulose membrane and blocked in 5% milk solution. Phosphorylated Cx43 (PCx43) was probed with anti phosphoCx43 (3511 Cell Signalling) at 1:1000 dilution. Goat anti-rabbit (Dako) at 1:5000 dilutions was used as the secondary antibody. Bands were developed using ECL solution and images obtained using chemidoc. The density of the bands was determined using imageJ software (http://rsb.info.nih.gov/ij/ .

Immunohistochemistry

Tissues were mounted with Tissue-Tek O.C.T (Sakura, USA) onto wooden blocks and 10µm sections obtained using the Leica CM1950 cryostat. Sections were picked up using poly-L-lysine coated slides and air dried. Sections were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1 % ($^{v}/_{v}$) triton X-100 for 20 minutes and blocked in a solution of 25 % ($^{v}/_{v}$) foetal calf serum in PBS for one hour. Primary antibody, anti phosphoCx43 (3511) diluted in 25 % ($^{v}/_{v}$) foetal calf serum in PBS solution were used to incubate the sections overnight at 4 ^{0}C in a black humid chamber. Sections were washed 3 times in PBS for 10 minutes each and a subsequent incubation in secondary antibody, goat anti- rabbit Alexa Fluor 488 (Invitrogen) simultaneously with wheat germ agglutinin (WGA), in the dark at room

temperature for one hour. Sections were washed in PBS and mounted with vector shield and visualised to obtain images using Zen LSM 710 confocal microscope, images were processed with ZEN lite software and the florescent intensity was determined using imageJ software.

Statistical Analysis

Data are expressed as means ± S.E.M and statistical differences assessed by Student's *t*-test using Microsoft Excel 2016. Results were considered significant when $p<0.05$. The number of samples or field of view used in each age group is defined by n.

Results

Heart/body weight ration in the test animals

The analysis of the body, heart weight and heart/body weight ratio at the age range considered in this study show no significant differences between the body weight of 6 and 24 month-old rats (671 ± 34.14 g and 589 ± 64.76 g) respectively (P> 0.05). Similarly, the heart weights were not significantly different (1.87 ± 0.09 g and 1.68 ± 0.16 g) respectively (P> 0.05), see table 1.

Table 1 Body weight, heart weight and heart/body weight ratio

Age of animal (n=10)	Body weight (g)	Heart weight (g)	Heart weight /Body weight ratio
6 months	671 ± 34.14	1.87 ± 0.09	2.8×10^{-3}
24 months	589 ± 64.76	1.68 ± 0.16	3.0×10^{-3}

PCx43 protein density and expression in the left ventricle

PCx43 protein density in the left ventricle significantly declined in the 24 months-old rats (figure 1 A & B). Similarly, the immunohistochemical images revealed a significantly reduced immunosignal in the 24 months-old rats when compared to the 6 month-old rats, (figure 2.A & B). Futhermore, there was a widespread remodelling of

the connexin expression in the 24 month-old rats, to a predominantly sparse punctate labelling at the intercalated disc, as against robust uniform labelling in the 6 month-old rats (See projected and magnified image in figure 2.A).

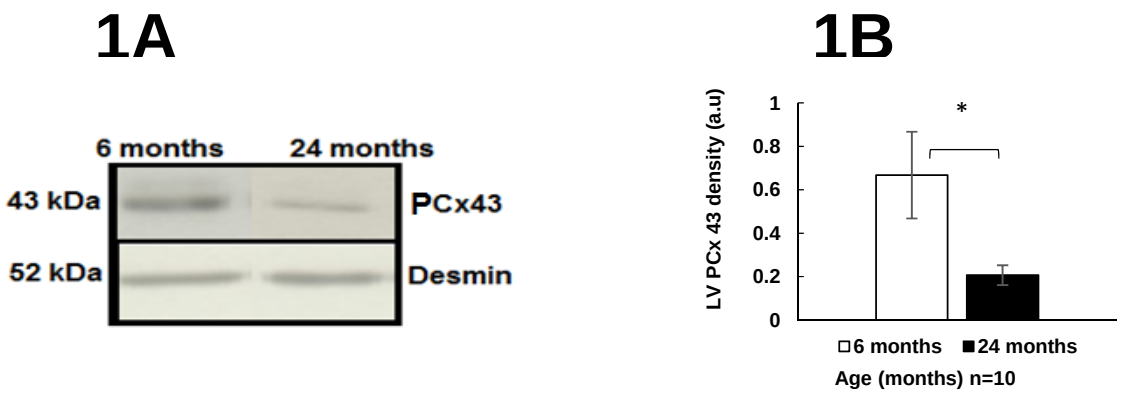


Figure 1 PCx43 protein density in the left ventricle of the Wister rats
A: Representative western blot of PCx43 protein in the left ventricle of the Wister rats .
B: Quantified PCx43 protein density in the left ventricle. PCx43 protein density and expression significantly declined in the left ventricle of the 24 month-old rats, P = 0.04 asterisk (*) signifies statistical significance. Error bars signify SEM.

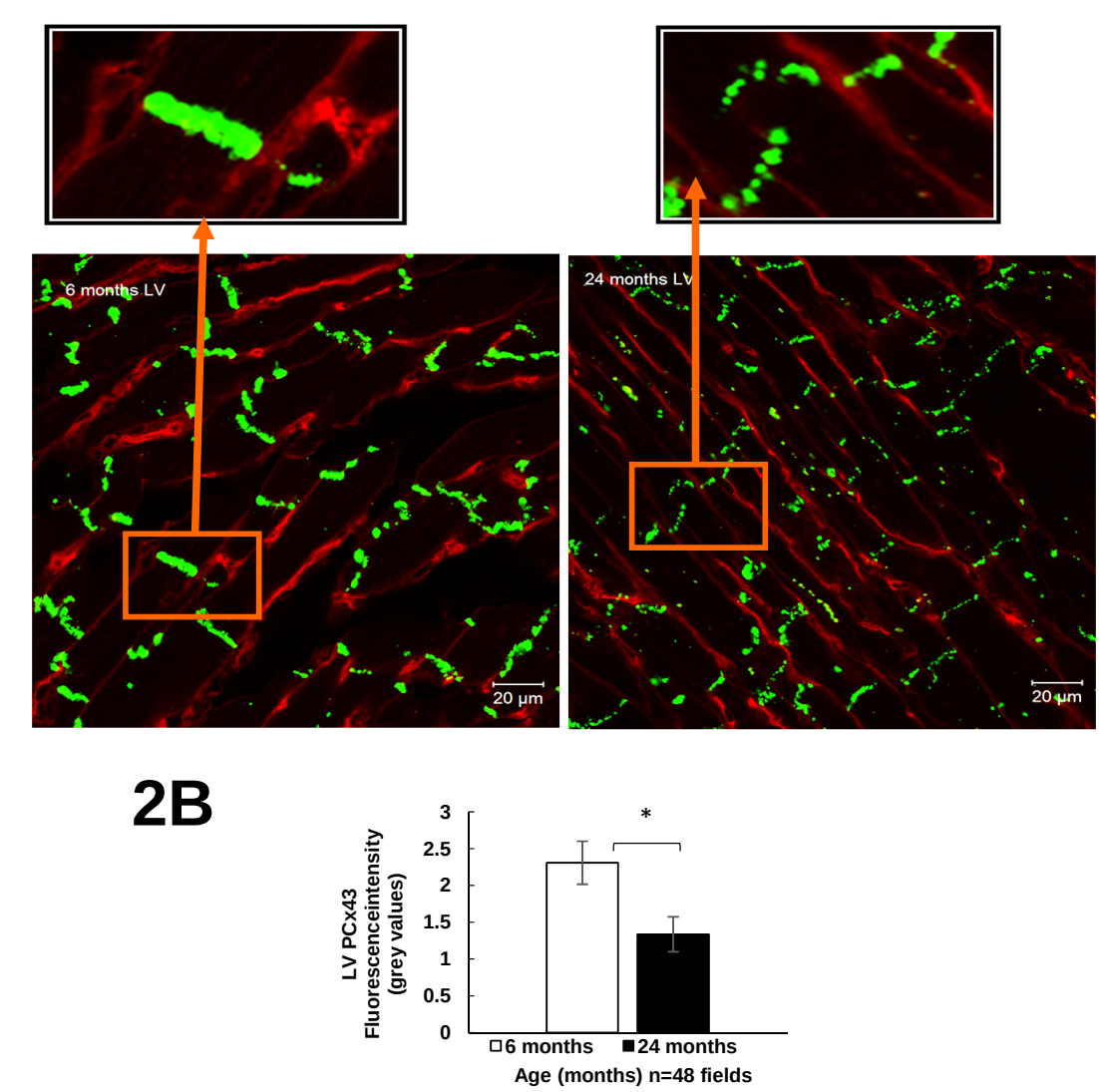
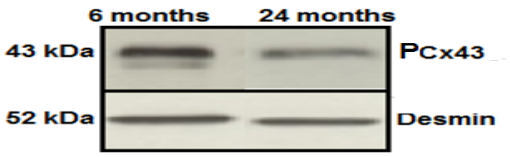


Figure 2 PCx43 protein expressions in the left ventricle of the rats. A: Representative immunofluorescence of PCx43 protein expression B: quantified immunofluorescence of PCx43 protein in the left ventricle of the rats. PCx43 protein density and expression significantly declined in the left ventricle of the 24 month-old rats (P value= 0.01). PCx43 appear as green fluorescent labelling at the intercalated discs, see magnified images.

PCx43 protein density and expression in the right ventricle

Using western blot, the PCx43 protein density showed a non-significant apparent decrease in the 24 month-old rats when compared to the 6 month-old rats (figure 3 A & B). Similarly, the quantified immunofluorescence signal was similar in both groups (figure 4.A &B).

3A



3B

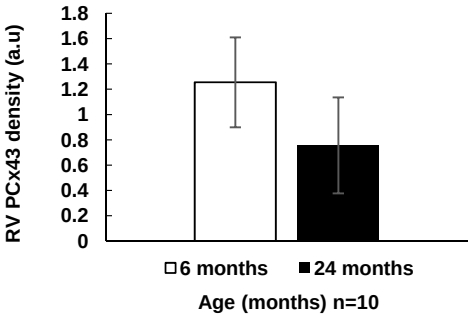
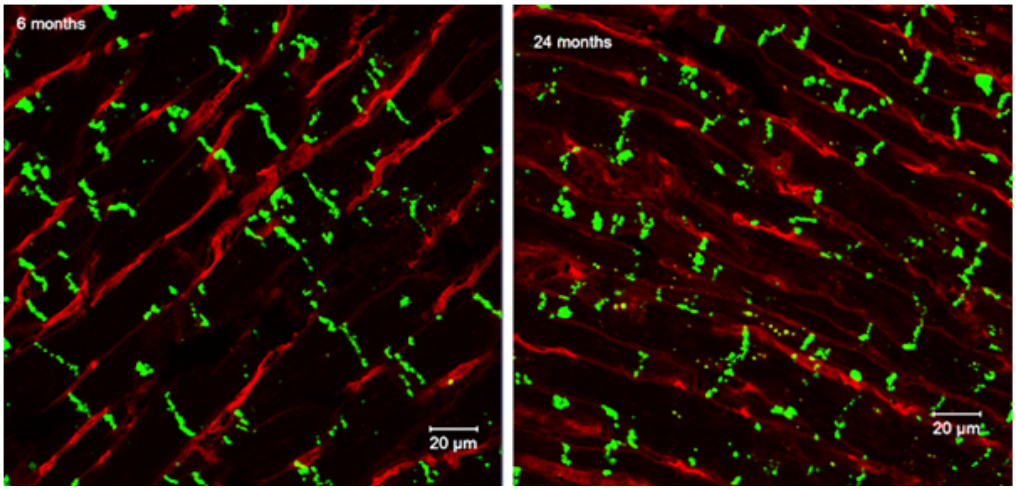


Figure 3 PCx43 protein density & expression in the right ventricle
A: Representative western blot of PCx43 protein density in the right ventricle.
B: Quantified PCx43 protein density. PCx43 density and expression did not significantly differ in 6 and 24 month-old rats (P=0.3).

4A



4B

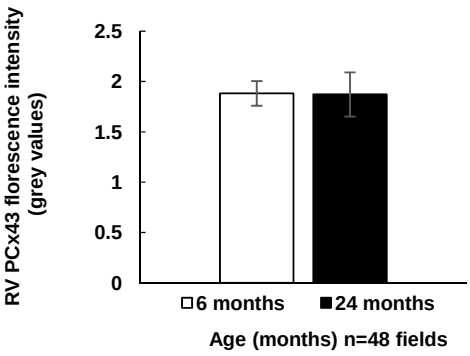


Figure 4 PCx43 protein expression in the right ventricle
A: Representative Immunoflorescence of PCx43 in the right ventricle.
B: Quantified immunofluorescence of PCx43. PCx43 expression did not significantly differ in 6 and 24 month-old rats (P= 0.9).

Discussion

The heart to body weight ratio of the animal models used in this study were similar, an indication that the animals were apparently healthy and that there were no cardiac hypertrophy in both animal groups. The phosphorylated Cx43 protein density and expression significantly declined in the left ventricle of 24 month-old rats. In addition, we noted a striking remodelling of PCx43 protein expression in the 24 month-old rats, from a robust homogeneous immunofluorescence as seen in the 6 month-old rats to a sparsely punctate labelling in the intercalated disc of the 24 month-old rats (see enlarged image in figure 2). Furthermore, there was some lateralization of PCx43 expression in the aged rats compared to the young. This finding is in support of the work of Lampe et al [23], who reported decreased phosphorylation, and increased lateralization [10 & 11] in arrhythmogenically remodelled hearts. However, the phosphorylated Cx43 density remained unchanged in the right ventricle of the two animal groups, our western blot analysis of right and left ventricles are supported by our immunohistochemical results.

Connexin43 can be phosphorylated at multiple serine residues [20, 25, 26, and 27], or either of two tyrosine sites [28] by different kinases. Phosphorylation at a particular site can affect connexin assembly, channel gating, trafficking and degradation [18, 22 29, 30 &31]. The phosphorylated Cx43 (PCx43) assay performed in this work measures connexins phosphorylated at S368 residue only. This phosphorylation site is a survival adaptation to ischaemic episodes [32]. It preserves intracellular coupling in a rat model [33, 29] following PKC activation prior to ischaemia. In addition, rotigaptide, a drug used to treat cardiac ischemia, acts by preserving phosphorylation at S368 and connexin coupling. In ischemic preconditioning, there is an activation of PKC which initiates a cascade of biological events culminating in increased phosphorylation at S368 and other events (that are yet to be fully understood) which constitute a defence strategy in a subsequent ischemic event [34].

The left ventricle is undoubtedly the region responsible for normal cardiac output and alterations related to reduced cardiac function, most often affect the left ventricle in the first instance and later, the right ventricle. Remodelling of the left ventricular PCx43 in the 24 month-old rats may signal a gradual progression towards a phenotype consistent with abnormal cardiac conduction. Altered connexin function is linked to changes of the electrical pathway of the heart, culminating in a change in

action potential propagation and cardiac conduction velocity. As such, changes in connexin expression pattern and decreased cardiac conduction velocity are principal features of cardiac arrhythmia and have been reported in heart failure [35, 36, and 9], chronic pressure overload and cardiac hypertrophy [37].

PKC phosphorylation of Cx43 has been reported to attenuate gap junction unitary conductance and decrease the likelihood of passage of death factor from an ischemic myocyte to a normal cell [27, 38 & 14]. The decline in the PCx43 in the left ventricle of the 24 month-old rats as reported in this work could imply a decreased tendency to orchestrate the survival strategy during episodes of local ischemia occurring in the left ventricle. This decline can be one of the factors that predispose the elderly myocardium to decrease cardiac conduction [39] and intracellular coupling, resulting from reduced and altered connexin43 expression leading to diseases of cardiac conduction defects which are more prevalent in the elderly.

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